THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY.

I. The Rôle of the Reticulo-Endothelial System in the Production of Diphtheria Antitoxin.

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INTRODUCTION.

The significance of the reticulo-endothelial system for the production of antibodies has recently aroused a great deal of interest. The early investigations into the production of antibodies which have been directed to the study of the leucocyte-forming organs, such as spleen, bone marrow, lymph nodes (1), or the glands of internal secretion, (testicle, thyroid) (2), as the source of the immune bodies, almost uniformly failed to prove any unmistakable relationship, although they did establish the fundamental importance of these organs in this process. More recent experiments on the reaction of the reticulo-endothelial system in immunity processes seem to point the way toward a better understanding of these hitherto obscure phenomena. It appears that the wide distribution of this particular tissue throughout the body and its general occurrence in the hematopoietic and lymphatic system, including the spleen and liver, would help to correlate the facts already observed and would also furnish a better explanation of many of the phenomena of general and local immunity.

Experience has shown that the reticulo-endothelial system, distinguished by Aschoff (3, 4) as a separate unit because of its ability selectively to take up and retain foreign particulate matter, can be effectively eliminated physiologically, in certain experimental animals, without inducing disproportionate, non-specific by-symptoms, by the intravenous injection of some indifferent colloid (such as India ink, various dyestuffs, or metallic salts). Many workers have used this blocking experiment in conjunction with splenectomy or without this procedure

as a criterion for determining the extent to which the reticulo-endothelial system participates in the production of the various antibodies. Subsequent to the early work of Murata (5), in 1918, as quoted by Aschoff (4), the significance of the reticulo-endothelial system, especially of its elements in the spleen, for the action of antigens in the body, was made the subject of extensive research by Bieling and Isaac (6). Experiments on the production of hemolysins in mice, in which these authors used the blockade together with splenectomy, suggested to them that the reticulo-endothelial system as a whole and, in particular, that part which is found in the spleen ranked foremost as the site of antibody production following the immunization with corpuscular antigenic material. Rosenthal and Fischer (7), in repeating this work with rabbits that were blocked and splenectomized, observed no inhibition of hemolysin formation, while Siegmund (8) found that the production of hemolysins and hemagglutinins was lessened or totally suppressed in highly blocked and splenectomized rabbits. Standenath (9), in testing precipitin formation in rabbits after the use of only moderate doses of India ink, found that it was increased rather than diminished, while splenectomized rabbits showed a lower titer. Another indeterminate observation was made by Fränkel and Grunenberg (10), who studied the formation of agglutinins against *proteus* X-19 in rabbits blocked with "elektroferrol." They found the agglutinin titer of the serum in blocked animals to be not materially lower than the titer of controls. Vannucci (11), on the other hand, reported the decrease of agglutinin formation subsequent to the injection of carmine and Wasserblau dye. Gay and Clark (12) in a recent paper report the average difference in the titer of hemolytic amboceptors produced in blocked rats and rabbits and in normal controls as 1:75 and assert that similar results are seen in precipitin formation. Paschkis (13) also regards the reticulo-endothelial system, especially that found in the spleen, as the site of antibody production and believes that a functional elimination of this system by blocking is possible. Kobayashi and Shiwotsu (14) studied the formation of typhoid agglutinins in rabbits which had been (*a*) injected with iron somatose, (*b*) splenectomized, (*c*) blocked and splenectomized. In agreement with Bieling, they found that agglutinin production was only partially inhibited by either blocking or removal of the spleen alone but practically no agglutination titer was observed in immunized rabbits in which blocking was combined with splenectomy. By following the response of treated animals to repeated injections of antigen, they found that a marked repair of the disturbed function took place, probably by compensation through other allied tissues. Neufeld and Meyer (15) observed that blocked and splenectomized mice frequently could not be actively immunized against pneumococci, while passive immunization in such animals is not interfered with. In a very interesting paper Singer and Adler (16) were able to show that a rabbit highly immunized against Pneumococcus Type III behaved just as a non-immune control animal, after the injection of India ink.

These various statements in the literature seem to indicate that by blocking the reticulo-endothelial system of experimental animals the

appearance of various antibodies (precipitins, agglutinins, hemolysins) was frequently prevented or delayed, which observations were still more distinct if the spleen was removed simultaneously, shortly before immunization.

In view of the somewhat conflicting reports in the literature, it is obviously important that further investigations in this field should be carried on. It would seem that the nature and dose of blocking material, method of administration, quality of antigen, species of experimental animal, and time interval between completion of the blockade and antibody determination, influence largely the outcome of the experiment. No work has as yet been reported in which the production of antitoxins in the blocked animal has been studied.

Walbum and Madsen (17) succeeded in increasing the antitoxic titer of the serum of horses by the intravenous injection of various metallic salts, and their results have since been confirmed by a number of other investigators. Neufeld (15) interprets these results as illustrating the opposite phase of the problem in question; namely, that very small amounts of colloidal material may induce an irritation rather than a blockade of the reticulo-endothelial system, with a subsequent increase of antibodies in the circulation (see also Standenath (9)). Experiments on the site of antibody formation would seem better grounded if the fate of antigenic material in the body were known to some extent. Wadsworth and Vories (18) have been able to show that neither the leucocytes of the dog nor those of the guinea pig neutralize or combine with diphtheria or tetanus toxin. Furthermore, they found in these experiments that, although brain tissue combines with and neutralizes tetanus toxin, as established by the work of Wassermann and Takaki (19), it has no action on diphtheria toxin. On the other hand, it appears from the work of Wadsworth and Hoppe (20) that diphtheria toxin and culture filtrates from a number of organisms had a definitely inhibitory action on the phagocytosis of leucocytes. This action was not affected by specific antitoxin nor antibacterial serums. This work clearly distinguished a substance which inhibited phagocytosis quite apart from the toxin—the toxin having no demonstrable action on these cells and suffering no loss of potency in their presence. Other workers have directed their attention to the hematopoietic organs, certain sessile cells of which are known to display a strong phagocytic activity. Their observations suggest that the bacterial toxins, such as diphtheria toxin and tetanus toxin, are fixed and absorbed by the spleen and apparently by the reticulo-endothelial system (Aschoff (3, 4), Bieling (21), Bieling and Gottschalk (22)).

A study of antitoxin production in animals in which the reticulo-endothelial system has been partially or wholly blocked would thus offer a promising field for investigation. Moreover, such a study

would have certain technical advantages as compared with the titration of other antibodies: the neutralization of diphtheria toxin by antitoxin, as carried out by the intracutaneous test, is delicate enough to show with sufficient accuracy any slight differences in the titer of antibody. Also, it would make possible the repeated determination of the antitoxin content in the tissues of the original experimental animal without recourse to tests in other animals or to *in vitro* titrations.

The occurrence of active and passive anaphylaxis in the blocked animal will be presented in a later paper.

EXPERIMENTAL WORK.

The study recently undertaken in this laboratory had for its object the comparison of diphtheria antitoxin production in guinea pigs, the reticulo-endothelial system of which had been blocked with India ink, with antitoxin production in normal, control animals, following a subcutaneous injection of diphtheria toxin-antitoxin mixture. To avoid repetition the first group will hereafter be referred to as blocked animals. By preliminary work it had to be determined, first, how much India ink could safely be given at one time to a guinea pig of a certain weight, and, second, how many injections had to be given to accomplish as complete a saturation of the reticulo-endothelial system as was consistent with the well-being of the animal. In much of the earlier work devoted to study of various factors in antibody formation, the animals were subjected to somewhat radical procedures (extensive surgical operations, injection of highly toxic substances, radiation, etc.) which in themselves constituted a serious interference with the physiological functions of life. In our experiments, we strove to attain an elimination of the reticulo-endothelial system by means of saturation with India ink that would not materially otherwise depress the vitality of the animal. It was found that the maximum dose of ink[1] that could safely be injected intravenously into a guinea pig of between 250 and 300 gm. weight was from 1.5 to 2 cc. of a 1:5 dilution with physiological salt solution. After such doses the

[1] The ink used was a commercial brand of India ink (insoluble) manufactured by Charles M. Higgins and Company, Brooklyn, New York.

animals remained perfectly well and did not show any symptoms of emaciation. By means of histological examinations it was found, furthermore, that two such injections following each other at short intervals would fill a large percentage of the reticular elements in the spleen and liver. These organs looked somewhat enlarged in the first days after these injections and had assumed an entirely black color. The bone marrow, also, looked grayish. Deposits of ink were found, besides, in the retrosternal lymph glands. Neither kidneys, nor adrenals, nor lungs seemed to be materially affected. In animals killed

TABLE I.

Determination of a Suitable Dose of India Ink for Blocking Injections.

Guinea pig No.*	Injections† (1:5 ink dilution).		Total volume injected.	Amount of concentrated ink injected.	Results.
	May 7	May 10			
	cc.	*cc.*	*cc.*	*cc.*	
1	2 2		4	0.8	Died in 2 hrs.
2	2 1		3	0.6	Very ill.
3	2	2	4	0.8	Slightly ill after second injection.
4	2	1.5	3.5	0.7	Very slightly ill after second injection.
5	1.5	1.5	3	0.6	No symptoms.
6	1.5	1.5	3	0.6	" "

* Animals weighed from 240 to 270 gm.

† Injections were given intravenously into the leg vein.

2 weeks after the completion of the blockade, the liver and spleen were very much reduced in size and were of hard consistency, apparently due to cirrhotic processes in which large areas of the parenchyma were replaced by connective tissue.

The method used for intravenous injections was that fully described in 1918 by Shiga (23) and later recommended by Roth (24, 25), who evidently had not seen Shiga's work on the subject, since he makes no mention of it in either of his two papers. Table I illustrates the finding of the maximum tolerance of guinea pigs for India ink.

After the dose of ink suitable for blocking had been thus determined, six guinea pigs, constituting the first experimental series, were blocked with two intravenous

injections of a 1:5 dilution of ink (1.5 to 2 cc.) on 2 different days; 2 days after the last blocking injection, they received 1 cc. of diphtheria toxin-antitoxin mixture[2] by subcutaneous injection. Simultaneously three normal, control guinea pigs were immunized with the same dose. After an interval of 3 weeks all nine animals

TABLE II.

Antitoxin Production in Maximally Blocked and in Normal Guinea Pigs Immunized with Diphtheria Toxin-Antitoxin Mixture.

Guinea pig No.	Weight of animals.	Blocking injections* (1:5 ink dilution).		Immunizing injections† (toxin-antitoxin mixture 140).		Results of 1st intracutaneous test Aug. 1.			Results of 2nd intracutaneous test Aug. 8.			Result of subcutaneous test Aug. 15.
		Date.	Dose.	Date.	Dose.	1/150 M.F.D.	1/100 M.F.D.	1/50 M.F.D.	1/150 M.F.D.	1/100 M.F.D.	1/50 M.F.D.	2 M.F.D. per 250 gm. body weight.
	gm.		*cc.*		*cc.*							
7	250	July 4	1.5	July 11	1	+	+	+	−	−	−	Survived.
		" 9	1.5									
8	250	" 4	1.5	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
9	260	" 4	2	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
10	300	" 4	2	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
11	300	" 4	2	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
12	240	" 4	1.5	" 11	1	+	+	+	−	−	−	"
		" 9	1.5									
13	260			" 11	1	−	−	+	−	−	−	"
14	250			" 11	1	−	−	+	−	−	−	"
15	275			" 11	1	−	−	Sl. +	−	−	−	"
16	260					+	+	+	+	+	+	Died.

* All blocking injections were given intravenously.

† All immunizing injections were given subcutaneously.

were tested by intracutaneous injections of 1/150, 1/100, and 1/50 M.F.D. of diphtheria toxin for the degree of immunity developed. Also, a normal guinea pig

[2] The mixture used was the 1/10 L+ dose of underneutralized diphtheria toxin-antitoxin mixture prepared and distributed as a routine product by this laboratory. This was used because it was a safer, quicker, and better known method of immunizing very susceptible animals.

that had been neither blocked nor immunized was tested as a control of the toxin dilutions. Readings were made each day for the first 3 days, then not again until the 5th and 7th days. A reaction was called positive only when a necrosis was observed on the 5th day. After another week, *i.e.* 4 weeks after immunization with the toxin-antitoxin mixture was begun, the intracutaneous injections were repeated on the other side of the animal. This method of testing the potency of toxin-antitoxin mixture follows closely the procedure described by Glenny (26)

TABLE III.

Antitoxin Production in Partially Blocked and in Normal Guinea Pigs Immunized with Diphtheria Toxin-Antitoxin Mixture.

Guinea pig No.	Weight of animals.	Blocking injections.*		Immunizing injections† (toxin-antitoxin mixture 140).		Results of 1st intracutaneous test Aug. 3.			Results of 2nd intracutaneous test Aug. 10.			Result of subcutaneous test Aug. 17.
		Date.	Dose.	Date.	Dose.	1/150 M.F.D.	1/100 M.F.D.	1/50 M.F.D.	1/150 M.F.D.	1/100 M.F.D.	1/50 M.F.D.	2 M.F.D. per 250 gm. body weight.
	gm.		*cc.*		*cc.*							
17	250	July 13	1.5‡	July 13	1	+	+	+	−	−	−	Survived.
18	240	" 13	1.5‡	" 13	1	−	+	+	−	−	−	"
19	260	" 13	2‡	" 13	1	−	+	+	−	−	−	"
20	250	" 13	2‡	" 13	1	−	+	+	−	−	−	"
21	275	" 11	1§	" 13	1	−	+	+	−	−	−	"
22	240	" 11	1§	" 13	1	+	+	+	−	−	−	"
23	280	" 11	1§	" 13	1	−	−	+	−	−	−	"
24	250	" 11	1§	" 13	1	−	+	+	−	−	−	"
25	250			" 13	1	−	−	+	−	−	−	"
26	260			" 13	1	−	−	Sl. +	−	−	−	"

* All blocking injections were given intravenously.

† All immunizing injections were given subcutaneously.

‡ A 1:5 ink dilution was used.

§ A 1:20 ink dilution was used.

and his associates. The series was concluded by testing all the animals after another week had elapsed, with a subcutaneous injection of 2 M.F.D. of diphtheria toxin per 250 gm. body weight for the establishment of general immunity. The results of this series, which are given in Table II, will be discussed below in connection with those of the following experiment.

Another series, consisting of eight guinea pigs injected with ink and of two normal controls, was run, with the object of studying in four animals the effect

of only one blocking dose (1.5 or 2 cc. of a 1:5 ink dilution), followed with practically no interval (from about 3 to 5 minutes) by the immunizing injection, and in the remaining four the effect of smaller doses of ink (1 cc. of a 1:20 ink dilution). Table III illustrates this work.

DISCUSSION.

Since the usual methods of investigating antibody formation depend necessarily upon the determination of the presence of antibody in the circulation or tissue fluid (which evidently also applies to intracutaneous tests), it is impossible with the tests employed to distinguish between the production of antibody in the cells of certain tissues and its presence in the body fluids. *A priori*, any inhibitory effect on the antibody titer, of blocking may be attributed to a delay in the appearance of the antibody in the circulation due to changes of permeability in the cell membranes as well as to a cellular underproduction of it. Conclusions as to the actual site of antibody formation, drawn from such experiments, are only indirect and, unless extensively controlled, can only bring out a more or less intimate causal relation of the reticulo-endothelial tissue to the production of immune bodies.

It appears from Tables I to III that, by giving a sufficient amount of India ink intravenously to guinea pigs shortly before immunization with diphtheria toxin-antitoxin mixture, a delay of antitoxin production for about 1 week, as compared with normal immunized controls, can be demonstrated. Tested 3 weeks after the immunizing injections, the blocked animals reacted, without exception, positively to intracutaneous injections of 1/150, 1/100, and 1/50 M.F.D. of diphtheria toxin, behaving at this stage just like non-immunized animals, while immunized unblocked control animals reacted positively only to 1/50 M.F.D. This deficiency in antitoxin production, however, is only temporary and is compensated for by another very quick rise in antitoxin production occurring in the following week, so that at the end of 4 weeks both blocked and non-blocked animals reacted negatively to 1/50 M.F.D. The degree of immunity eventually attained in both groups is similar, as evidenced by the survival of all animals after the subcutaneous injection of 2 M.F.D. From comparison of the two series it must be concluded that for the depression of antitoxin production the dose of blocking material is of greater importance than the duration of the interval between blocking and immunization.

Finally, and contrary to Neufeld's supposition, it was shown that the small doses of ink employed in these experiments (about 1/12 of the blocking dose used in the first series) did not have a stimulating effect, but proved likewise to exert some inhibitory action on the production of antitoxin. This failure of smaller ink doses to exert a stimulating effect might be due to the fact that the doses used were not sufficiently small to bring about the physiological irritation.

The authors realize that the results obtained in this study must be interpreted with reserve in view of the fact that the antigen used was a complex substance, the action of which in inducing active immunity may not be as simple a process as that of toxin alone.

CONCLUSIONS.

1. Following massive doses of India ink injected intravenously into guinea pigs before a subcutaneous injection of diphtheria toxin-antitoxin mixture, no antitoxin was found in the blood serum for 3 weeks, as indicated by intracutaneous tests, whereas an appreciable amount could be detected in non-blocked, immunized control animals.

2. During the 4th week following immunization, the titer of the serum of blocked animals equaled that of non-blocked controls within the limits of the intracutaneous test dose.

3. The smaller doses of India ink used in these experiments, given before immunization, had no stimulating effect on the production of diphtheria antitoxin but, on the contrary, also inhibited the appearance of this antibody, although to a less extent.

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